IN VITRO AGROBACTERIUM MEDIATED TRANSFORMATION AND REGENERATION OF WHITE CLOVER (*TRIFOLIUM REPENS* L.)

By

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White clover is a high quality forage that fixes atmospheric nitrogen and can improve animal fertility. Genetic transformation technology has the potential to enhance existing varieties and to be used in the development of novel varieties for future use. A genotype independent tissue culture regeneration system is required to use these genetic technologies and this has proven to be a limiting factor for white clover. A new regeneration protocol based on germinated seedlings was tested on five white clover cultivars adapted to the USA. This system proved to be much less genotype specific than previous reports, with 39 to 51% of the individuals in these five cultivars producing 1 or more shoots. Attempts to use this system in combination with *Agrobacterium*-mediated transformation techniques, however, proved fruitless. A different regeneration protocol

was investigated that used imbibed seeds as the source of tissue for the cultures. Results from this system showed equally high levels of prolific regeneration. The use of this protocol with *Agrobacterium* proved successful and transgenic white clover plants were recovered at a frequency of ca. 4%. PCR and Southern blot data provided molecular evidence of integration of the novel genes in the transformed regenerated plants. Hand pollination between transgenic and non-transgenic plants produced progeny which segregated at the expected 1:1 ratio, which indicated the stable sexual transmission of the novel genes. A greenhouse test was conducted to evaluate the response of transgenic and non-transgenic plants to levels of the herbicide phosphinothricin (PPT). Results indicated that some transformed plants were completely resistant to 200 mg/L PPT and could tolerate 2000 mg/L while other transformants could not resist 200 mg/L. Use of this protocol from a practical breeding point of view and concerns due to intellectual property rights are also addressed.

INTRODUCTION

Plant genetic engineering is rapidly becoming a practical tool for cultivar development and improvement. The first decade of research primarily involved the development and optimization of proven transformation protocols for major experimental and agronomic plant species. Improvements in vectors to integrate the chimeric DNA, along with more effective selectable marker genes for recovering transgenic plants, has made it possible to transform many different plant species. Refining transformation protocols for greater convenience, higher efficiency, and broader genotype range is still a high priority in some species. The following dissertation describes the requirements and methods for a white clover (*Trifolium repens* L.) *Agrobacterium* transformation protocol. The objectives of this experiment were to : (1) evaluate the effectiveness of genotype independent direct shoot regeneration protocols using white clover explants from cultivars adapted to the southeast United States (2) utilize the most effective regeneration protocol to develop a white clover *Agrobacterium*-mediated transformation protocol in which both transformed and non-transformed plants with the same genetic background are recovered *in vitro*. The importance of recovering the non-transformed control is discussed. Initially the transformation protocol was based on Voisey et al. (1994) and later changed to a modified Larkin et al. (1996) protocol.

LITERATURE REVIEW

White Clover

White clover (*Trifolium repens* L.) is the most important pasture legume in many parts of the temperate zones (Carlson et al. 1985). In addition to being a high quality forage, white clover promotes growth of associated grasses and improves soil fertility by fixing atmospheric nitrogen. Other benefits of white clover in pastures include improvements in animal health, milk flow, calf weaning weight, daily gains, and conception rates (Carlson et al. 1985). White clover is considered very nutritious and palatable at all stages of development. All kinds of livestock, including dairy and beef cattle, sheep, hogs, and poultry, relish the tender, succulent leaves. Cultivars of white clover can be classified as small, intermediate, or large (Ladino), essentially the same except for size of leaf, degree of flowering and plant height. White clover is usually planted with one or more grass companions to reduce the risk of bloat in sheep and cattle, increase the chance of a good stand, and aid in mowing and curing of hay.

Although a perennial, in the southeast United States white clover stands commonly decline within two to three years after establishment (Dobson et al. 1976). Virus diseases and nematodes have been suggested as the major factors in the weakening of white clover

plants, thus making them more susceptible to injury and death from other environmental stresses and diseases (Gibson et al. 1981).

Breeding and Genetics

White clover ($2n$=4x=32) is an outcrossing species with disomic inheritance. A strong gametophytic self-incompatibility system based on multiple oppositional alleles at the *S* locus exits in white clover (Williams 1987). It is characterized by independent action of S alleles in both pollen and style. Growth of pollen tube carrying a given S allele is arrested in stigmas bearing an identical allele. Populations are, therefore, a heterogeneous mixture of highly heterozygous individuals, resulting in high levels of genetic variation both within and between populations. The large amount of genetic variability present in white clover populations/cultivars enhances overall persistence in competitive, grazed swards, where a wide variety of micro-environments are encountered. The goal of a white clover breeder in developing cultivars is to increase the frequency of favorable genes while avoiding inbreeding depression. Phenotypic recurrent selection within adapted germplasm pools has been by far the most common means of population improvement in white clover (Williams 1987). Improved production from grazing animals and persistence are major breeding goals. Cultivars released to date have been synthetic varieties produced from open pollination of selected elite clones or seed lines. These populations are maintained by random pollination in isolation.

Regeneration

A regeneration system independent of genotype is desirable for a highly out-crossing species like white clover. White clover is responsive to a wide range of regeneration protocols. Plants have been regenerated from callus (Mohapatra and Gresshoff 1982; White 1984; Bhojwani et al. 1984; Webb et al. 1987a; Yamada 1989), protoplasts (Bhojwani and White 1982; Webb et al. 1987b; Yamada 1989), directly from stolon segments (Bond and Webb 1989), and through somatic embryogenesis (Parrot 1991; Weissinger and Parrott 1993). All of these regeneration protocols were limited, however, to very few genotypes with high regeneration capacity. White and Voisey (1994) reported a method to regenerate shoots directly from cotyledonary stalk tissue of germinated seedlings that reduced the strict dependency on genotype. White and Voisey (1994) split the cotyledon in half and obtained numerous direct shoots with an average of 20/cotyledon. Beattie and Garrett (1995) obtained adventitious shoot production from immature embryos of single cross *T. repens* cultivar Haifa plants using a technique that also may be independent of genotype. Most recently, Larkin et al. (1996) obtained high frequency direct shoot regeneration from the same cotyledon-hypocotyl region using pre-germinated, water-imbibed seeds.

Plant Transformation

In order to successfully introduce foreign DNA into the plant nuclear genome, many different tools are required. A desirable gene for agronomic or experimental

purposes, promoters, selectable markers, and an efficient vector for stable DNA integration are a few of the essential tools. The next few sections will describe in more detail the components required for white clover transformation. Although there are many different options for what components are combined in an overall transformation protocol, only tools that were used in this study or may be valuable for future white clover transformation experiments will be described. Currently single gene traits are most commonly inserted into a plant genome. Virus, insect, and nematode resistance are desirable traits that may be used to increase stand persistence in white clover. White clover transformed with a herbicide resistance gene could enable new weed control options for broad leaf weeds in clover pastures. Less common, but also desirable, are genes that alter quality or produce substances that are too complex or expensive for laboratory synthesis.

Virus Resistance

Plant virus infection can cause severe damage, resulting in significant yield reduction. Since there is no known way to completely eradicate a virus from commercial production of many agronomic crops, a number of preventative strategies are typically implemented. Some of these strategies include planting certified virus free cultivars, extensive spraying of chemical insecticides to eliminate vectors for transmission, and breeding for host plant resistance. Of these strategies, host plant resistance is the only feasible option for a forage legume such as white clover. Breeding for resistance to viruses, however, is normally complicated because resistance genes are either unknown or found in wild-type germplasm. Extensive backcross programs are usually required to

eliminate other undesirable wild type genes that are linked to the resistance genes. This can be very expensive, require many years to complete, and the occurrence of resistance breaking virus biotypes is always a possibility. Clover yellow vein virus, peanut stunt virus (PSV), and alfalfa mosaic virus are the predominant viruses affecting white clover in the southeast United States (McLaughlin et al. 1992). *Trifolium ambiguum* may contain genetic resistance to peanut stunt virus (Pederson and McLaughlin 1989), however, resistance to the most common viruses including PSV is lacking in domesticated germplasm (Pederson and McLaughlin 1994). The expression of virus-derived genes in plants, either in a functional or mutated form, can protect the host plant from the virus in which the genes were isolated. This appears to overcome many of the drawbacks of breeding for resistance. With the advent of improved cell culture and molecular biology techniques, virus genes can be introduced and expressed in a wide variety of crops without interfering with important agronomic traits. The virus coat protein (CP) gene, the virus replicase gene, and movement protein (MP) genes are the most common sequences used for transgenic protection. Integration of virus gene in the antisense orientation or mutating the sequence to render the mRNA transcript untranslatable or the mature protein non-functional are common resistance strategies. The coat protein gene of PSV has been cloned (Naidu et al. 1991) and may be useful for inducing stable long-term resistance in white clover. There is currently no model that explains how coat protein derived resistance is achieved. Gene silencing via RNA:RNA hybridization or an *in vivo* dsRNA degradation system that is only active when a certain threshold of dsRNA is achieved are current theories (Goodwin et al. 1996; Tanzer et al. 1997; Jorgensen et al. 1998; Wassenegger and

Pelissier 1998). Interference of the invading virus life cycle by a mutated virus protein to stop replication or movement may be involved in some resistance situations.

The Bt Gene(s) and Insect Resistance

Insect pests are a major cause of damage to the world's important agricultural crops. Common control strategies include the application of chemical pesticides to eliminate the pest. Integrated pest management (IPM), a more environmentally sensitive approach, uses multiple control measures to reduce the insect damage below the economic threshold. Trap crops, resistant cultivars, crop rotation, spraying, and insect phermones are frequently used techniques for IPM control. The use of resistant varieties is an effective control method, however, genes for insect resistance are not always available or easily integrated into existing cultivars. The development of insect resistant biotypes along with multiple insect pests can quickly lead to major crop damage. A new strategy for insect control involves transforming plants with a resistance gene(s) from another species that conveys resistance. Most of the insecticidal genes currently in use are of bacterial origin, in particular from *Bacillus thuringiensis* (*Bt*). The soil microorganism *Bt* has proven to be a rich source for insecticidal proteins and genes. During the sporulation phase, *Bt* produces parasporal crystals that consist of about 130 kDa proteins know as δ-endotoxins (Koziel et al. 1993). They exert their toxicity by binding to the midgut epithelial cells and ultimately causing osmotic lysis through pore formation in the cell membrane (Gill et al. 1992). *Bt* has been used as an insecticide for more than 40 years, but it was the cloning and sequencing of the insecticidal protein genes (Schnepf and Whiteley 1981) that raised the

prospects of using insecticidal proteins in transgenic plants. Early experiments indicated that the gene was expressed in plants but at a level too low to convey adequate field resistance. The gene was modified by increasing the GC content of the sequence for increased plant expression. Currently, there are a few cultivars available that exhibit high levels of insect resistance by expressing a *Bt* gene (Estruch et al. 1997). Known *Bt* strains contain a great variety of δ-endotoxin encoding genes. A total of 96 genes have been described so far and more are being reported routinely. Based on recent USA patents that have been filed, some of these genes appear to convey high levels of resistance against the major root knot nematodes that infest root systems of some important crops including white clover.

Quality Enhancement

Most of the transgenic plants currently in production were developed for insect, virus, or herbicide resistance. Other possibilities are altered plant fats and oils, methionine- and lysine-enhanced grain and legume proteins, plant foods that can deliver immunizing antigens, and alteration of metabolic processes such as fruit ripening (Day 1996). White clover already possesses a high protein content and many of the enhancements previously mentioned are catered toward human consumers. Since it is a perennial and produces high levels of vegetative growth per unit time, it may make an ideal species for pharmaceutical or biodegradable polymer production (Haq et al. 1995; Nawrath et al. 1995). Bioremediation by expressing genes for heavy metal and mercury accumulation may be possible with white clover. It could be beneficial to transform white clover with gene(s)

that are involved with synthesis of an organic molecule or protein that is too complex for commercial laboratory production. In this way the active ingredient could be produced in the leaves and extracted at multiple harvests throughout the growing season.

An Elite Cultivar to Transform--Osceola

Osceola is a ladino-type, medium-blooming white clover cultivar adapted to Florida and other white clover producing areas of the United States (Baltensperger et al. 1984). It was developed over a 30 year period to provide a longer-lived, more productive, and better reseeding ladino-type for Florida conditions. Seeds from 35 selected intercrossed clones were bulked in 1973 to produce the breeder's seed for the synthetic cultivar Osceola white clover. Osceola combines better summer stolon persistence and fall regrowth with sufficient seed production to ensure a stand.

Vector for DNA Integration

Assuming a desirable gene is available, a vector to integrate the foreign DNA into the plant nuclear genome is required. There are many published protocols that describe a novel way to integrate the DNA. These methods may or may not be reliably reproduced or feasible for a variety of plant species. *Agrobacterium* and a biolistic approach are the most common and reproducible methods for DNA integration used to date. The biolistic approach is literally a shot gun approach in which the foreign DNA is coated onto micro size gold or tungsten beads. The coated beads, propelled by a helium gas blast and regulated by rupture disks, are directed at plant tissues that can be regenerated (Sanford

1988). The biolistic approach is best suited for monocots and dicotyledons species that are recalcitrant to *Agrobacterium* transformation.

The *Agrobacterium* - plant cell interaction is the only known natural example of DNA transport between kingdoms (Sheng and Citovsky 1996). *Agrobacterium tumefaciens* is a soil plant pathogen that genetically transforms host plant cells. Wild type strains of *Agrobacterium* transfer genes that code for plant hormone-like compounds that cause rapid undifferentiated cell division resulting in a tumor-like structure known as a gall. Another set of genes that code for amino acid-like molecules termed opines are also integrated into the plant genome. These genes function in association to provide a place for the invading bacteria to live, the gall, and food to ingest, opines. Other soil microorganisms can not metabolize opines, therefore, creating a favorable biological niche for *Agrobacterium*. The DNA that is transported from the bacteria (T-DNA) and inserted in the plant genome is located on a large tumor inducing (Ti) plasmid. T-DNA is defined by two 25-bp imperfect direct repeats known as the left and right borders. All other genes required for transfer of the T-DNA to the plant nucleus, known as the virulence (*vir*) genes, are also located on the Ti plasmid. Seven major *vir* loci (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G, and *vir*H) and a few genes located on the *Agrobacterium* chromosome (*chv*) are involved in the chain of events from excision of T-DNA to binding of bacteria to host plant cell surface receptors, to integration of T-DNA into plant genomic DNA. Table 1 contains a summary of major events and genes involved in the T-DNA transfer process. To exploit *Agrobacterium* as a vector for plant transformation, the wild type bacteria must be disarmed by removing the wild-type tumor and opine genes. Because the T-DNA is

defined by its borders, the coding region can be replaced by a DNA sequence without a deleterious effect on transfer from *Agrobacterium* to the plant. Original vectors introduced the chimeric gene between the left and right T-DNA boarders of the Ti plasmid by homologous recombination. It was later discovered that the *vir* genes will work *in trans* and that the T-DNA region could be located on a separate plasmid. The binary vector system (Hoekema et al. 1983), uses two compatible plasmids, one containing the *vir*-region, the other carrying the T-DNA on a wide host-range replicon. This system has many advantages over homologous recombination. The Ti plasmid is large and difficult to manipulate. By placing T-DNA on a separate, smaller plasmid, it can be easily genetically manipulated using *Escherichia coli* as a host. Transfer of this plasmid into an *A. tumefaciens* strain harboring the the disarmed Ti plasmid with the *vir*-region allows introduction of manipulated T-DNA into plant cells. Binary plasmids are usually 10-20kb with the T-DNA region somewhat smaller. Recent evidence indicates that use of a binary bacterial artificial chromosome vector, with helper plasmids enhancing production of VirG and VirE proteins, can allow efficient *Agrobacterium* mediated transfer of at least 150kb of foreign DNA into the plant nuclear genome (Hamilton 1997). This may allow large regions of chromosomes that are thought to contain certain genes or quantitative trait loci to be transformed into the plant genome.

Agrobacterium Strains and Plasmids

Different strains of *Agrobacterium* can vary greatly in their virulence to specific plant species. Inoculating a wounded plant with a variety of wild type *Agrobacterium*

strains and then evaluating tumor formation is a common way to determine if a particular strain is a good candidate for transformation. Many confirmed virulent wild-type strains have been engineered to disarm the T-DNA and enhance virulence. *Agrobacterium* strains EHA101 and AGL1 are both derived from wild type A281, a L,L-succinamopine strain (Hood et al. 1986). A281 contains a C58 chromosomal background and is hypervirulent on several solanaceous plants. The Ti plasmid of A281, pTiBo542, was disarmed producing strain EHA101. The disarmed Ti plasmid of EHA101 was further modified to produce AGL1 (Lazo et al. 1991). Both EHA101 and AGL1 are hypervirulent *in trans* when a binary plasmid containing the T-DNA borders is present. Table 2 contains a summary of how the *Agrobacterium* strains were derived and what binary plasmid they contain.

The *Agrobacterium* plant transformation vector is most commonly exploited using a binary vector approach. The binary plasmid that contains the T-DNA borders may be quite variable. The genes that are located between the left and right border and their arrangement are common differences. The T-DNA is thought to be transferred in a right border to left border, 5' to 3', orientation (Zambryski 1988; Gleave 1992; Becker et al. 1992). Selectable marker genes are, therefore, better located closest to the left border making it the last gene to be integrated, assuming complete T-DNA transfer. If T-DNA transfer from the plasmid is prematurely aborted and only partial T-DNA starting from the right border is integrated into the plant genome, resistant cells lacking the novel gene will not be recovered. If the selectable marker gene is closer to the right border than the novel gene and T-DNA transfer is aborted prematurely, plants may be recovered that express the

selectable marker gene but lack the novel gene. Other differences between binary plasmids include their size in base pairs, restriction sites available for cloning, and which antibiotic resistance genes they carry for maintenance of the binary plasmid.

Binary vector plasmid pCP001 contains a 1764 bp tobacco (*Nicotiana tabacum* cv. Samsun) basic chitinase promoter sequence derived from FB7-1 (Neale et al. 1990). This had been PCR-engineered to allow cloning into the *Hind*III and *Bam*HI sites of the binary transformation vector pBI101.3 (Clonetech, Palo Alto, CA). The FB7-1 promoter:β-glucuronidase:nopaline synthase terminator (chitinase:*gus*:NOS) cassette was excised with *Eco*RI and *Hind*III. *Eco*RI linkers were added, and the cassette was cloned into a unique *Eco*RI site in the binary transformation vector pTAB10 (Kahn et al. 1994) to create pCP001 (Figure 1). This plasmid also contains a 35S:*bar* selectable marker.

Binary vector plasmid pMON9793 (McKently 1995) is a derivative of pMON505 (Monsanto Co., St. Louis, MO) in which a chimeric gene containing a mannopine synthase promoter(MAS), the coding region for β-glucuronidase (*gus*) (Jefferson 1987), and the nopaline synthase (NOS) 3' polyadenylation signal was cloned into the multilinker (Rogers et al. 1987). Plasmid pMON9793 also contains a chimeric neomycin phosphotransferase II (NPTII) selectable marker gene with the NOS promoter and NOS 3' polyadenylation signal.

Promoters

Plant promoters are regulatory elements and constitute one of the major factors that determines the temporal and spatial expression of a plant gene(s). A promoter can be

constitutive and allow for transcription in all cells, or it can be tissue specific and only transcribe the gene in that particular cell type. Strong promoters induce high levels of transcription producing high levels of steady state mRNA, in contrast to weak promoters that result in low levels of mRNA synthesis. Eukaryote promoters contain conserved regions in the 5' leader sequence. These include a CAAT and TATA box which aids in recognition and of the promoter and binding by transcription factors (TF's). These TF proteins are essential because RNA polymerase II cannot bind directly to eukaryotic promoter sites and initiate transcription without their presence. RNA polymerase II can initiate transcription from a promoter *in vitro* in the presence of five factors: TATA-binding protein (TBP), TFIIB, TFIIE, TFIIE, TFIIF, and TFIIH (Emili and Ingles 1995). Fusing the beta-glucuronidase (*gus*) reporter gene (Jefferson 1987) to an uncharacterized promoter and then assaying enzymatic GUS activity is a common way to study promoter specificity and strength. This can be misleading, however, due to post-transcriptional processes that may result in gene silencing, although the promoter is fully active and functioning. Early promoters used for transformation experiments were thought to be constitutive were and used to control transcription of selectable marker genes. Now with advancements in tissue specific promoter isolation, along with more desirable agronomic foreign genes that require specific temporal expression, many new plant promoters are available. The following sections contain a description of all promoters used in this study.

Agrobacterium tumefaciens, the wild type plant pathogen, carries eukaryotic-like promoters that, when integrated into the host plant genome, transcribe genes for amino acid-like molecules termed opines. Opines serve as a food source for the invading bacteria.

The promoters for two opine production genes, mannopine synthase and nopaline synthase, were some of the first promoters used in plant transformation experiments. Initially it was thought that these promoters were constitutive, thus making them good candidates to control transcription of selectable marker genes, detectable marker genes, or any other gene that should be transcribed in all cells.

Mannopine synthase (MAS)

The promoter for mannopine synthase, originally thought to be constitutive, is now considered inducible and, in some cases, tissue specific. Strongest expression is usually seen in the phloem and roots. This promoter is also wound-inducible, which increases transcription and alters tissue specificity, leading to expression in leaves (Guevara-Garcia et al. 1993). The addition of the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA) also enhanced expression (Saito et al. 1991).

Nopaline synthase (NOS)

In nopaline-type tumor tissues, the nopaline synthase gene (*nos*) is one of the most abundant transcripts (Dai and An 1995). Since the *nos* gene was considered to be constitutively active in a variety of plant tissues, the promoter has been used to control transcription of plant selectable and detectable marker genes. It was later shown, however, that the *nos* promoter activity is organ-specific and developmentally regulated (An et al. 1988). In seedlings, lower parts exhibit a higher activity compared to upper parts. In older plants, the promoter activity is very low throughout the entire plant except in roots and

certain reproductive organs (An et al. 1988). Promoter activity is enhanced or induced by wounding, auxin (An et al. 1990), H_2O_2 (Dai and An 1995), methyl jasmonate and salicylic acid, many of which are thought to be involved in plant defense related signal transduction pathways (Kim et al. 1993).

Cauliflower mosaic virus 35s (35s)

The 35S promoter region of the cauliflower mosaic virus (CaMV) provides a model plant nuclear promoter system, since its double-stranded DNA genome is transcribed by host nuclear RNA polymerase II from a CaMV minichromosome (Oiszewski et al. 1982). When the 35S promoter fused to a chloramphenicol acetyltransferase reporter gene was transformed into tobacco, expression was approximately 30 times stronger than the nopaline synthase promoter (Nagy et al. 1985). Moreover, it was constitutively expressed in all organs of transgenic plants. The relatively high promoter strength and constitutive type of expression has made the 35S promoter a good candidate for controlling the transcription of chimeric genes used as selectable markers in plant transformation experiments.

Tobacco basic chitinase

The tobacco basic chitinase gene was isolated when Meeks-Wagner et. al (1989) were attempting to clone genes transcribed specifically during flower development in tobacco. A RNA differential display approach to identify mRNA that is transcribed in early floral but not vegetative meristems was implemented. A thin cell layer (TCL) *in vitro*

tissue culture system that could be induced through hormones to develop into vegetative or floral meristems was used for the poly A mRNA source of early genes in floral development. Floral bud transcripts at day seven (FB7) were isolated from three different gene families. FB7-1, the first group of transcripts isolated, were detected in stem and internode segments and leaves of plants possessing an immature inflorescence. The highest levels of FB7-1 transcript were found in the roots, reaching a maximum level at the stage immediately prior to the transition of the shoot apex to its reproductive state (Meeks-Wagner et. al 1989). Using DNA sequence data, Neale et al. (1990) determined that the FB7-1 sequence belonged to the chitinase gene family. In tobacco, this gene family comprises both acidic and basic chitinases (Legrand et al. 1987). All FB7-1 sequences show extensive homology to the basic chitinase sequence isolated from tobacco Havana 425 (Shinshi et al. 1987). White clover cv. Haifa, an intermediate type, and tobacco cv. Samsun, were transformed with the tobacco basic chitinase promoter fused to the *gus* reporter gene to compare the temporal and spatial expression of this promoter between species (Pittock et al. 1997). Transcription from the promoter was induced by similar developmental and environmental signals in each species. In white clover, no staining was observed in leaf, floral or stem tissue unless mechanically wounded. The strongest expression was in root meristems of all main and emerged lateral root tips. This root specific expression was dramatically reduced during the period of floral initiation and flowering.

Selectable Marker Gene

A selectable marker gene is a key requirement for any plant transformation protocol. This gene must be constitutively transcribed in the initially transformed cell(s). It must then be expressed in all further cell divisions and ultimately in the entire plant. The selectable marker gene codes for a protein that can detoxify, degrade, or inhibit a phytotoxic chemical. After the DNA integration step, the plant material is placed on selection medium containing the phytotoxic chemical in order to eliminate non-transformed cells and permit the growth and proliferation of only transformed cells. An ideal selectable marker gene should not permit plant material to escape the selection screen, resulting in recovery of plants that do not express the desired transgenes. Two main classes of selectable marker genes have been used in plant transformation experiments. One class encodes proteins that confer resistance to antibiotics such as kanamycin and hygromycin B (Waldron et al. 1985). The other class encodes proteins that confer tolerance or detoxify a herbicide. Resistance to atrazine (Cheung et at. 1988), glyphosate (Comai et al. 1985), and sulfonyl-urea herbicides (Haughn et al. 1988) have been achieved by the introduction of foreign genes encoding modified insensitive target proteins. Resistance to phosphinotricin (PPT) (DeBlock et al. 1987), bromoxynil (Stalker et al. 1988), and 2,4-dichlorophenoxyacetic acid (2,4-D), (Streber and Willmitzer 1989) are based on expression of detoxifying enzymes originally isolated from microorganisms. Selectable marker genes vary in their effectiveness depending on which gene is used (Witrzen et al. 1998), the plant species in which it is expressed, and the concentration needed to kill non-transformed cells.

Since the selectable marker gene is integrated into genomic DNA, these genes are active throughout the life of the plant and in progeny. The nature of these genes, antibiotic / herbicide resistance, and their persistence is a major reason for some groups to reject the use of plant transformation technology. The next section will describe the two plant selectable marker genes used in this study.

NPTII

The most commonly used selectable marker is the gene from transposon 5 (Tn5) from *Escherichia coli* K12 encoding aminoglycoside 3-phosphotransferase II (APH(3') II). This enzyme commonly known as neomycin phosphotransferase II (NPTII), inactivates kanamycin and neomycin by phosphorylation (Flavel et al. 1992). Kanamycin inhibits protein synthesis by targeting the ribosome and interfering with translocation and elicits miscoding. The original gene housed in a large segment of Tn5 DNA was sequenced (Beck et al. 1982) and an open reading frame that codes for a 264 amino acid protein was confirmed to be the exact NPTII gene. This gene acts as a dominant selectable marker when transformed into eukaryotic cells. To facilitate the safety assessment of the NPTII protein, the same coding sequence used for plant transformation was introduced into *Escherichia coli* to produce gram quantities of this protein (Fuchs et al. 1993a). The NPTII protein was shown to degrade rapidly under simulated mammalian digestive conditions (Fuchs et al. 1993b). It was concluded (Fuchs et al. 1993b) that ingestion of genetically engineered plants expressing NPTII protein poses no safety concerns and Flavel et al. (1992) comment that overall, the ubiquity of the gene in nature and its benign

properties make it ideal as a selectable marker in plant transformation. NPTII is the selectable marker gene on plasmid pMON9793.

bar

Bar and *pat* are similar genes with the same function. The bialaphos resistance gene (*bar*) confers resistance to the commercial herbicide bialaphos and codes for a phosphinothricin acetyltransferase (PAT). The *bar* gene, which shows significant sequence homology to the *pat* gene, was isolated from *Streptomyces hygroscopicus* (Thompson et al. 1987). The *pat* gene which codes for a PAT protein was cloned from *Streptomyces viridochromogenes* Tu494 (Strauch et al. 1988). Bialaphos and phosphinothricin (PPT) are potent non-selective herbicides. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus*. It consists of PPT, an analogue of L-glutamic acid, and two L-alanine residues. In both bacteria and plants, intracellular peptidases remove the alanine residues and release active PPT. PPT is a potent inhibitor of glutamine synthase (GS). This enzyme plays a central role in the assimilation of ammonia and the regulation of nitrogen metabolism in plants (Miflin and Lea 1977). It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation, and photorespiration. Inhibition of GS by PPT causes rapid accumulation of ammonia which leads to death of the plant cell (Tachibana et al. 1986). PPT, also known as glufosinate, is chemically synthesized (BASTA, Finale™, LIBERTY™, IGNITE®, RELY®), while bialaphos is produced by fermentation of *Streptomyces hygroscopicus* (Herbiace, Meiji Seika Ltd.). To summarize, both the *bar* and *pat* genes encode a phosphinothricin

acetyltransferase (PAT), which acetylates the free NH_2 group of PPT, thereby preventing autotoxicity in the producing organism (Murakami et al. 1986). Therefore, in addition to its value as a selectable marker, *bar*'s ability to confer resistance to PPT makes it a useful gene for production of herbicide resistant plants. The *bar* gene is the selectable marker on plasmid pCP001.

Detectable Marker - beta-glucuronidase (*gus*)

Detectable marker genes, more commonly known as reporter genes, are very useful for plant molecular biology studies. Fusion of the reporter gene to an uncharacterized promoter offers an effective way to study gene regulation. If the reporter gene tolerates amino terminus fusion and can pass across membranes, it can be used to study protein transport and organelle targeting (Jefferson 1987). Other factors related to post transcriptional and post translational processing may interfere with quantification of the reporter gene and should be accounted for if possible. By using a reporter gene that encodes an enzyme activity not found in plants, the sensitivity with which chimeric gene activity can be measured is limited only by the properties of the reporter enzyme and the quality of the available assays for the enzyme (Jefferson 1987). Some older plant reporter genes include neomycin phosphotransferase, chloramphenicol acetyl transferase (Herrera-Estrella et al. 1983), nopaline synthase (Depicker et al. 1982) and firefly luciferase (DeLuca and McElroy 1978). Difficulty in assaying or high endogenous activity are some reasons why these genes are seldom used at this time. The *E. coli* beta-glucuronidase (*gus*) gene (Jefferson 1987) is the most popular plant reporter system currently implemented.

GUS is a hydrolase that catalyzes the cleavage of a wide variety of beta-glucuronides, many of which are available commercially as spectrophotometric, flourometeric and histochemical substrates. GUS is very stable, and will tolerate many detergents, widely varying ionic conditions, and general abuse. GUS has no cofactors or ionic requirements, although because it is inhibited by some heavy divalent metal ions, EDTA should be added when assaying. Detection of GUS activity depends on the availability of substrates for the enzyme which, when acted on by the enzyme, liberate a product that is distinguishable from the enzyme. The substrate should be cleaved only by the enzyme under study with minimal spontaneous cleavage. Although GUS has fluorogenic substrates for better quantitative analysis, only the histochemical substrate used in this experiment will be described. The best substrate currently available for histochemical localization of GUS activity in tissue and cells is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The substrate gives a blue precipitate at the site of enzyme activity. The product of GUS action on X-Gluc is not colored. Instead, the indoxyl derivative produced must undergo an oxidative dimerization to form the insoluble and highly colored indigo dye. The dimerization is stimulated by atmospheric oxygen, and can be enhanced by using oxidation catalyst such as K+ ferricyanide/ferrocyanide mixture. Without such a catalyst, the results are often very good and Jefferson (1987) suggested infrequent use of a catalyst for routine work. The *gus* gene is fused downstream from the tobacco basic chitinase and MAS promoter in plasmids pCP001 and pMON9793, respectively.

DNA Anaylsis

Southern blot

This technique, first reported by Southern (1975), enables one to detect the presence of a certain DNA sequence (target) based on the complementarity between the target and a labeled DNA fragment known as the probe. The method is used to detect a particular DNA fragment in a mixture of DNA fragments such as those obtained when genomic DNA is digested with a restriction endonuclease. After restriction, DNA is fractionated by gel electrophoresis. The gel is then treated to make the DNA single stranded and then the DNA is transferred to a nylon membrane by capillary action. The hydrogen bonding sites on the nitrogenous bases in the single-stranded genomic DNA are available to hydrogen bond with the single-stranded probe. The probe labels are generally separated into two classes: radioactive and non-radioactive. An isotope frequently used to label probes is phosphorous 32 esterified to the C5' of deoxyribose. The decay of the radioisotope within the probe emits energy in the form of beta particles which identifies the location of the probe when detected by exposure of the membrane to X-ray film. This allows for determination of the presence and size of a particular genomic DNA fragment that is complementary to the probe. Most of the non-radioactive labeling and detection systems utilize enzyme linked probes (e.g. biotin, alkaline phosphatase or horseradish peroxidase) and a chemiluminescent substrate to induce an *in situ* chemiluminescence that is detectable with X-ray film.

Polymerase chain reaction--PCR

The polymerase chain reaction (Mullis and Faloona 1987; Saiki et al. 1988) is a technique used to amplify a specific sequence of DNA known as the target. A thermostable DNA polymerase, nucleotides, Mg+, DNA primers, and a thermocycler are the components required for the amplification reaction. The theory for the reaction is as follows: (1) high temperature to denature the double stranded DNA containing the target sequence (2) optimized temperature for DNA primers to anneal to target sequence (3) DNA synthesis at the required temperature for the thermostable polymerase (4) Repeat 1-3. Millions of copies of the target sequence can be generated when the reaction is repeated multiple times. The amplified product is then run on a electrophoresis gel and viewed by ethidium bromide staining.

White Clover Transformation

Previously, White and Greenwood (1987) described a white clover transformation system dependent on a highly regenerable genotype, WR8. In this system, stolons were incubated with *Agrobacterium*. The very low frequency of transformation and the strict genotype dependence made this approach impractical. White and Voisey (1994) split 3-day old cotyledons in half and obtained numerous direct shoots with an average of 20/cotyledon, however, Voisey et al. (1994), in the same journal issue, did not use the split cotyledons, instead using intact cotyledons to achieve *Agrobacterium*-mediated transformation of white clover with a frequency of less than 1%. Transgenic plants

appeared to be recovered from the apical meristem and not as direct shoots from the cut region. Furthermore, in another publication, this group reported white clover transformed with the pea albumin gene using genotype WR8 and the older stolon transformation system (Ealing et al. 1994). Most recently, Larkin et al. (1996) combined their imbibed seed regeneration protocol with *Agrobacterium*-mediated transformation and regenerated transformed plants at a frequency varying from 5-80% with the *bar* gene. The efficiency of transgenic plant recovery was consistently an order of magnitude better with PPT than with kanamycin selection.

Table 1. Summary of cellular process that occur between *Agrobacterium* and a plant cell

Cellular process	Agrobacterium role	Agrobacterium genes involved
Cell-cell recognition	Binding of Agrobacterium to the host cell surface receptors	ChvA, ChvB, PscA, Att
Signal transduction	Recognition of plant signal molecules	ChvE, VirA, VirG
Transcriptional activation	Expression of vir genes after phosphorylation of the transcriptional activator	VirG
Conjugal DNA metabolism	Nicking at the T-DNA borders and mobilization of the transferable single stranded copy of the T-DNA (T-strand)	VirD1, VirD2, VirC1
Intercellular transport	Formation of protein-DNA T-complex; formation of a transmembrane channel; export of the T-complex into the cytoplasm of the host plant cell	VirE2, VirE1, VirD2, VirD4 VirB4, VirB7, VirB9 VirB10, VirB11
Nuclear import	Interaction with the host cell NLS receptors and transport of the T-complex through the nuclear pore	VirD2, VirE2
T-DNA integration	Integration into the plant cell genome; synthesis of the second strand of the T-DNA	

Table 2. *Agrobacterium* strains used in white clover transformation experiments.

Strain	Genotype and / or Bacterial Phenotype	Binary Plasmid
EHA101	C58 pTiBo542 T-region::*aph*, Km^R	pMON9793
AGL0*	EHA101 pTiBo542DT-region Mop^+	
AGL1	AGL0 *recA::bla* pTiBo542DT Mop^+ Cb^R	pCP001

* Strain AGLO was not used but was included here to indicate how AGL1 was formed

Abbreviations : pTiBo542 - hypervirulence; aph aminoglycoside phosphotransferase

Km^R - kanamycin resistance; Mop - mannopine utilization; Cb^R - carbenicillin resistance

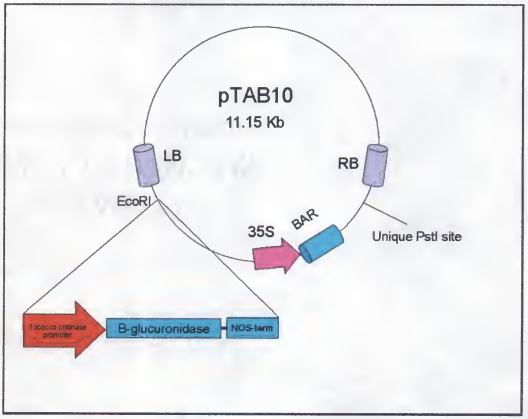

Figure 1. Map of plasmid pCP001 used with Agrobacterium strain AGL1 for white clover transformation.

MATERIALS AND METHODS

White Clover Regeneration

Germinated Seedlings Preparation

Seeds of four white clover cultivars adapted to the southeastern U.S.A ('Osceola', 'Regal Ladino', 'California Ladino', 'Lousiana S1') and one experimental population (Florida Red Leaf) were surface-sterilized by submerging them into 50% sulfuric acid for 2 minutes followed by four, 1-minute washes in glass distilled deionized water. Seeds were then plated on 0.8% (w/v) gum agar (Sigma Chemicals Co., St. Louis, MO.) and incubated at 26°C; 16-h photo period, to germinate. A five-day incubation period was required to achieve the proper seedling stage (Figure 2). Explants were obtained by making an excision at the dark green band that separates the hypocotyl from the apical portion of the seedling containing the two cotyledons and an extremely small apical meristem (Figure 2). Explants were plated horizontally on shoot inducing medium. Split explants were obtained in like manner with the addition of a second symmetrical excision resulting in a pair of cotyledons still containing their stalk and a small portion of the hypocotyl. Individual cotyledons were plated with the abaxial surface in contact with the medium Fifteen different media containing MS salts (Murashigi & Skoog 1962), and B5 vitamins

(Gamborg 1968), 30 g/L sucrose, 8 g/L agar formulated from five levels of 6-benzylaminopurine (BAP) (0.1, 0.25, 0.5, 1.0, 2.0 mg/L) and three levels of α-naphthaleneacetic acid (NAA) (0, 0.05, 0.1 mg/L) were tested for shoot inducing efficiency by counting number of shoots per explant at 21 days afer plating.

In experiments to determine the optimum shoot inducing medium and to evaluate for cultivar differences in induction, explants were not split and twenty explants were plated per dish. Four replications, each containing 900 genotypes, were evaluated. To determine the effect of the second excision resulting in a pair of cotyledons still containing the hypocotyl, 160 split and 208 intact cv Osceola explants were compared on the medium found to be optimum in the shoot induction experiments.

Water Imbibed Pre-germinated Seeds

Seeds of cv Osceola were surface sterilized in 0.5% sulfuric acid for 30 seconds followed by four, 1-minute washes in sterile water. Seeds were left in the last wash and incubated for 15 hours at 15°C in the dark. Using a binocular microscope, explants were obtained by making a single slice in the seed coat with a #10 blade and removing the two separate cotyledons still containing a small piece of the hypocotyl region by squeezing. Explants were plated abaxial side down on B5 medium (Gamborg 1968), containing 20 g/L sucrose, 7 g/L agar, 12 nM Picloram and 2.2 mM N^6-benzylaminopurine. One week after plating, explants were reoriented so the hypocotyl region was in good contact with the medium. A random sample of 100 cv Osceola seeds (200 cotyledons) was evaluated by observing shoot induction 21 days after plating.

Data Collection & Analysis

Twenty-one days after plating on shoot inducing medium, direct shoots were counted on all explants using a binocular microscope. Only shoots originating from the cut end of the explant (opposite the apical end) were counted. An analysis of variance (ANOVA) using a completely randomized design was performed on the four replications in the germinated seed protocol to detect cultivar and medium differences for shoot induction.

White Clover Transformation

Preliminary Lethality Tests

A few preliminary tests were conducted to determine if kanamycin, carbenicillin, or PPT effected shoot induction. Carbenicillin was tested on both split and intact germinated cotyledons at 300 mg/L. Thirty eight dishes each containing 11 genotypes were evaluated. Four levels of kanamycin, 0, 25, 50, 100 mg/L were tested for effect on shoot induction on non-split germinated cotyledons. Fifty genotypes per/treatment, replicated twice, were evaluated. PPT at 10 mg/L was tested on 24 non-transformed imbibed genotypes. PPT was also tested at 20 mg/L in the rooting medium on 24 non-transformed explants that contained many vigorous green shoots without roots.

Medium Preparation

Recovering both a transformed and non-transformed plant from the same genotype required four different mediums (Table 3). Basal medium powder stocks for shoot induction (SH) and root induction (RI) were purchased from Sigma. All hormones, antibiotics, and PPT were filter sterilized and added after autoclaving. Medium was stored at 8°C. Media were prepared and used within a two-week period.

Seed Sterilization - H_2SO_4, PPM

Initially a tea strainer was used to contain seeds while being sterilized. With this method, the strainer containing white clover Osceola seeds was submerged into 50% H_2SO_4 for 1 minute, followed by four 1 minute washes in sterile water. Seeds were incubated in the dark at 15°C for 16 hours and rinsed once in sterile water immediately prior to use. The protocol was revised by eliminating the strainer and reducing the percent of H_2SO_4. Seeds were poured into a beaker containing 0.25% H_2SO_4 for 30 seconds followed by four 1-minute sterile water washes. Seeds were incubated in the dark at 15°C for 16 hours and rinsed in water immediately prior to use. The sterilization procedure was later changed to a PPM (plant preservative mixture; Plant Cell Technology, Inc, Washington, D.C.) based method. PPM contains antibacterial and antifungal properties. Seeds were poured into a beaker containing 1 ml/L PPM and incubated in the dark at 15°C for 15 hours. Seeds were used directly from the PPM solution.

Cutting the Explant

Since the co-cultivation tray used contained 24 wells, individual experiments contained 24 genotypes. A binocular scope with a fiber optic light source for enhanced visibility, two dishes of SH medium, a large number of imbibed seeds and sterilized tools were arranged in the laminar flow hood. Initially petri dishes were labeled on the bottom with a felt tipped marker to indicate explant number (1-24). For speed and convenience, a generic template made out of transparency film containing a laser printed 1-24 spot grid was developed. The template was placed under the petri dish providing a uniform grid for explant placement and identification. It was essential that the seeds were at the ideal stage for explant removal; imbibed but not ruptured. The two cotyledon explants were obtained the same way as described in the regeneration section. A #10 scalpel and a very thin spatula were used to handle and transport individual cotyledons. The first cotyledon from the first genotype was plated in a specific numbered location on SH medium. The other cotyledon was plated in same location on the other SH dish. One dish of 24 cotyledons was labeled with a test number and incubated to induce shoots. The other plate containing the 24 cotyledon partners was used for *Agrobacterium* co-cultivation (see below).

***Agrobacterium* Growth and Storage**

Agrobacterium strain EHA101 was originally grown by placing a single colony from an agar streak (Figure 3A) into 20 ml Luria-Bertani broth (LB) supplemented with 100 mg/L spectinomycin and 50 mg/L kanamycin. The flask was placed on an orbital

shaker at 300 rpm overnight at room temperature. Cells were measured at optical density 620 nm (OD_{620}) to determine the concentration using 5 X 10^8 cell / ml for 1 OD_{620}. Four flasks of bacteria at OD_{620} 0.9 - 1.2 were centrifuged at 2000 x g for 10 min and the pellets re-suspended in 80 ml 10 mM $MgSO_4$ supplemented with 100 uM acetosyringone. After many spectrometer readings, a distinct color (Figure 3B) and aroma were characteristic of bacteria at OD_{620} 0.8 - 1 and all further bacteria cultures were not read with the spectrometer. This protocol was adjusted by using 10 ml LB and removing the centrifuge and resuspension step. *Agrobacterium* strain AGL1 was grown in a similar manner using 20 mg / L tetracycline. Table 4 summarizes the recipes for solid and liquid phase bacteria growth. Long-term storage of bacteria was achieved by adding 5% (v/v) DMSO to log phase liquid cultures and dispensing 1 ml into small centrifuge tubes. Tubes were frozen at -80°C. Approximately one year later, a frozen stock of strain AGL1 was tested by streaking on fresh LB medium supplemented with the appropriate antibiotics.

Co-Cultivation

Initial transformation experiments used the re-suspended *Agrobacterium* strain EHA101 and germinated split cotyledons. The bacteria were loaded into a 10cc syringe (Figure 3C) and a single drop was placed at the cut region of the explant. The explant was co-cultivated with the bacteria for three days on shoot inducing (SH) medium and then rinsed and transferred to shoot inducing medium for selection (SH+). With the imbibed seed method using strain EHA101:pMON9793 and AGL1:pCP001, the bacteria were applied in the same way, covering the entire cotyledon. Ultimately, strain AGL1:pCP001

was dispensed into a Costar® 24 well cell culture cluster (Figure 3D), at a rate of 1 ml/well. One of the two cotyledons from a single genotype was then placed into a filled well. After all wells were filled, the container was covered and periodically agitated for 1 hour. Explants were then removed, blotted on filter paper and plated on (SH) medium. Three days later the explants were rinsed in sterile water, blotted and plated on (SH+) medium.

Recovering Plants

Seven stages, starting with seed preparation were required to recover rooted plants in the greenhouse (Figure 4). Three days after placement of non-transformed explants on SH medium, explants were reoriented by pushing the enlarged hypocotyl into the medium. Explants that were co-cultivated were transferred to new SH+ medium 10-14 days after initial plating. After well established green shoots were present, plantlets on SH and SH+ were transferred to root inducing media with and without selection (RI+, RI). Explants were left on RI and/or RI+ until a main and lateral root tips were present. Rooted plants were transplanted into individual peat pellets and incubated in a plastic mist box with 24 hours of flourescent light at 70°F. When many healthy roots tips were seen protruding through the side of the pellet, plants were transplanted into 6 inch plastic pots and placed on concrete blocks on the floor of the greenhouse. A large piece of wood supported by two benches was placed over the plants to block out any direct sunlight. After two days, plants were moved onto the greenhouse benches in direct sunlight.

Evaluation of Transformed Plants

As soon as green transformed shoots can be putatively identified in stage one, a variety of confirmation assays can be performed. These can continue through the first cross seed generation or any later generation seed related to the original transformed parental genotype. The different assays that can be implemented depends on what genes were integrated into the plant genome. Only assays that can be performed with plasmid pCP001 and pMON9793 will be described.

X-Gluc Stain Preparation

It was previously mentioned that 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) is a histochemical substrate for the *gus* gene product. Although many literature citations indicate that this stain was formulated with all components including catalysts to enhance dimer formation, the author discovered that in actuality many researchers do not include these catalysts (GA Moore, M. Gallo-Meagher, personal communication). For this experiment, X-Gluc stain was generated by dissolving 5 mg of 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt into 50 ul N,N-dimethyl formamide. A solution containing 2.5 ml 0.2 M $NaPO_4$ and 100 ul 0.5 M Na_2EDTA was added to the dissolved X-Gluc. Subsequently, 2.4 ml D.I. H_2O was added to obtain a volume of 5.0 ml. The stain was then transferred to five micro-centrifuge tubes each containing 1.0 ml and used immediately or frozen for storage.

GUS Petiole Histochemical Assay

This test was performed after shoot induction when sufficient plant material was available to sacrifice and not limit opportunity to recover complete plants. The assay required one to five, half-millimeter petiole slices and was performed by initially adding 45 ul X-Gluc stain to empty 1.5 ml micro centrifuge tubes. Petiole slices were then inserted into tubes (1 tube/genotype), making sure that all petiole tissue was fully submerged into the stain. At least one transformed and non-transformed control tube, if available, were also included. All tubes were placed in the dark at 37°C for 12 to 24 hours. Tissues were cleared of chlorophyl to aid in stain detection by pipetting out X-Gluc stain and adding 70 ul 3:1 ethyl alcohol (95%) : glacial acetic acid. The clearing step was repeated if tissue was not adequately cleared in two hours. Stained tissue was recorded immediately after the incubation period, however, it was noticed that the tissue could be stored at room temperature in the clearing agent for months.

GUS Root Histochemical Assay

This test was best performed with plants containing the tobacco basic chitinase promoter fused to the GUS reporter gene because it had been shown to produce main root tip and lateral root tip specific expression (Pittock et al. 1997). Small root tips were excised from plants rooting in PPT or by carefully removing them from the sides of plants rooted in peat pellets. The assay required one to three two-millimeter lateral or main root tip cuttings. Forty-five µl X-Gluc stain were added to empty 1.5 ml micro centrifuge

tubes. Clean root tips were inserted into the tubes insuring that the tip end was fully submerged in the stain. At least one transformed and non-transformed control tube, if available, were included. All tubes were incubated in the dark at 37°C for 12 to 24 hours. Stained tissue was recorded immediately after the incubation period, however, as with petioles, the root tips could be stored at room temperature in the clearing.

Leaf Painting Assay

This assay was best performed on plants in the greenhouse that contained at least three green healthy young leaves. The assay tests for effectiveness of the *bar* gene and, therefore, requires plants transformed with plasmid AGL1:pCP001. On a sunny day, crossing labels were attached to single leaflets indicating the date of application. A small paint brush was used to apply 20 mg/L glufosinate (PPT) to the adaxial side of the leaf, making sure that at least one or two drops of herbicide remained adhered to the leaf surface. Five to seven days after application leaves were observed for herbicide damage.

Herbicide Application Tests

To determine how well the 35S promoter fused to the *bar* gene functions in white clover, a replicated herbicide resistance test was performed. A randomized complete block design was used to test for differences in PPT resistance. Four treatments using the pure ammonium salt form of glufosinate (PPT) at 0 mg/L, 20 mg/L, 200 mg/L and 2000 mg/L were sprayed on both transformed and non-transformed partners. Multiple plants of each genotype were vegetatively propagated from each plant produced *in vitro*. Each block

contained all treatments and was replicated twice. Data were collected as a plant health rating of 0 (completely resistant) to 5 (completely dead) at seven days after PPT application. Analysis of variance (ANOVA) was used to test for differences in PPT resistance between transformed and non-transformed partners and between different transformed genotypes.

Somaclonal Variation or Insertion Effect

Aside from gross phenotype differences such as mutant morphology or color alterations, whole plant differences between transformed and non-transformed partners will likely be hard to detect. In the herbicide application test, each block contained each transformed and non-transformed partner genotype in quadruple. Prior to spraying PPT, the two replications provided eight clones at a similar stage of development for evaluation. Data were collected as a plant health / vigor score (1 -5); 1 weak and poor growth to 5 healthy and vigorous growth.

Crossing and Segregation of Transgenes

Controlled crosses were made in both directions between transformed plants and either a white clover plant with the dominant red leaf mark (Red Leaf) or a four leaf variety with the dominant red leaf mark (Four Leaf). Seeds of individual flower heads were germinated in metal trays and grown to a young stage when the red leaf mark could be clearly identified . All plants were sprayed with 50 mg/L PPT and counts for number of resistant and susceptible plants were taken after one week.

The χ^2 analysis was used to test for disomic inheritance of a single copy T-DNA insertion in the T_1 generation (1:1).

Southern Blot

White clover genomic DNA was extracted using the method of Dellaporta et al. (1983) that utilizes a SDS (sodium dodecyl sulfate), potassium acetate precipitation of proteins and carbohydrates. Both plant leaves dehydrated overnight (Tai and Tanksley 1990) and liquid nitrogen frozen leaves were used for DNA extraction. Approximately 25 µg of DNA was digested with highly concentrated (70 units/µl) *Eco*RI which cuts the introduced T-DNA fragment once to yield unique fragment sizes. Undigested DNA and DNA cut with *Eco*RI and *Pst*I to release the intact *bar* gene were also included. Digested genomic DNA and a purified 0.60-kb *bar* sequence was separated by gel electrophoresis (0.8% agarose) at 33 volts for 18 hours. DNA was transferred to Hybond-N nylon membrane (Amersham, Arlington Heights, IL) by capillary action and then cross-linked to the membrane by exposure to UV. Blots were prehybridized for 5 hours at 65°C in a $NaPO_4$ buffer containing 7% SDS (sodium dodecyl sulfate), BSA, and 100 ug/ml sonicated/denatured salmon sperm. The probe consisted of a gel purified 0.60-kb *Pst*I fragment of the *bar* coding region labeled with [^{32}P]dCTP by the random primer technique according to the Prime-a-Gene protocol (Promega Corp., Madison, WI). Hybridization was performed at 65°C for 20 hours and then washed three times for 20 minutes with 0.1X SSC. The blot was exposed to X-ray film for 7 and 14 days.

Polymerase Chain Reaction

A 418 bp internal sequence of the *bar* gene was amplified using two 18-nucleotide primers homologous to the upper and lower DNA strands (upper: 5' ^{57}GGCGGTCTGCACCATCGT^{74} 3', lower: 5' ^{458}GCCAGTTCCCGTGCTTGA^{475} 3'). Amplification conditions using Taq polymerase were as follows : denaturation, 94°C for 1 min; annealing, 60°C for 1 min; extension, 72°C for 1.5 min. Reactions were run for 30 cycles. PCR products were analyzed on a 1.2% agarose gel.

Table 3. Plant growth medium used to induce shoots and roots in non-transformed and transformed partner genotypes.

Non - Transformed		Transformed	
SH	B5 basal salts and vitamins (Gamborg et al., 1968) 0.7% Agar (Sigma gum) 2% Sucrose pH 5.8 Autoclave 2.2 uM BAP 12 nM Picloram	SH+	Same as SH with Autoclave 20 mg / L PPT 250 mg / L Cefotaxime
RI	M & S w/ B5 vitamins 0.8% Agar (Sigma gum) 2% Sucrose pH5.7 Autoclave 1.2 uM IBA	RI+	Same as RI with Autoclave 20 mg / L PPT 250 mg L Cefotaxime

SH=Shoot Inducing : RI=Root Inducing

Table 4 Medium used for liquid and solid phase *Agrobacterium* growth.

Strain	EHA101 : pMON 9793	AGL1 : pCP001
Solid	LB Agar - pH 7 100 mg / L Spectinomycin 50 mg / L Kanamycin	LB Agar - pH 7 20 mg / L Tetracycline 20 mg / L Rifampicin
Liquid	LB Broth - pH 7 100 mg / L Spectinomycin 50 mg / L Kanamycin	LB Broth - pH 7 20 mg / L Tetracycline

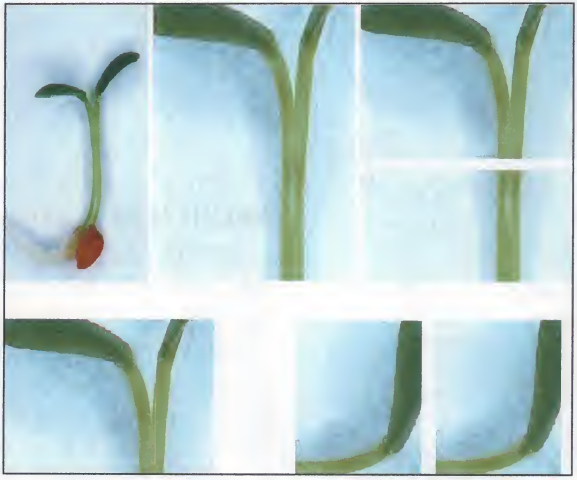

Figure 2. The Three day old seedling and how to obtain the intact and split cotyledons for tissue culture.

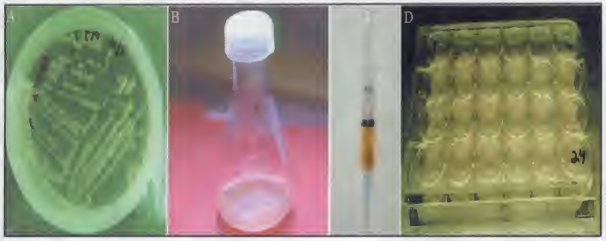

Figure 3. Solid and liquid phase Agrobacterium growth.
A) Streak of single colony bacteria; B) Flask containing log phase bacteria; C) Syringe used For micro-drop co-cultivation; D) Co-cultivation 24 well container.

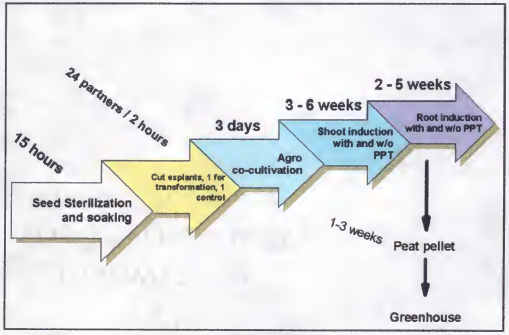

Figure 4. Time frame required to regenerate transformed plants.

RESULTS

White Clover Regeneration

Germinated Seedlings

The analysis of variance revealed significant differences (α=.05) for media effect on direct shoot induction. Medium containing 0.05 mg/L NAA and 1 mg/L BAP was most effective at inducing direct shoots, however, two of the remaining fourteen media tested were not significantly different for shoot induction (Figure 5). The least numbers of direct shoots were produced on media supplemented with 0.1 mg/L BAP. Increasing the concentration of BAP increased the number of direct shoots up to 1 mg/L. This trend was not observed at 2 mg/L BAP, which produced results similar to 0.25 mg/L. Direct shoots appeared to originate from the cut end of the explant after a swelling phase, approximately 20 days after placement on shoot inducing medium (Figure 6). In some cases, shoots were also observed emerging from the apical end prior to the formation of direct shoots. Genotypes in all cultivars produced from 0 to 17±3 shoots/explant. The percentage of genotypes producing one or more direct shoots ranged from 39% for Florida Red Leaf to 51% for California Ladino. Mean number of shoots per explant ranged from 1.6 for Red Leaf to 2.3 for California Ladino. Only Louisiana S1 (1.8) and Red Leaf (1.6) differed

significantly (α=.05) from other entries according to Duncan's new multiple range test (Table 5). The additional splitting step used to initiate cultures reduced the number of un-responsive explants by 50%, and increased the number of genotypes that produced more than ten shoots per explant from 8.6% to 20% (Figure 7).

Water Imbibed Pre-Germinated Seeds

One hundred percent response was obtained after the cutting technique had been mastered. Although this method was not statistically compared with the germinated seed protocol, it appeared to have a 100% explant response rate for direct shoot induction and explants producing greater than ten shoots per explant (personal observation). Individual explants produced clusters of direct shoots all originating from the cut end oposite the apical shoot meristem region. Two weeks after plating most explants contained greater than 15 shoots, with some cotyledons producing more then 50 shoots (Figure 8).

White Clover Transformation

Preliminary Lethality Tests

No significant difference (α = 0.5) in shoot induction or proliferation could be detected on medium supplemented with carbenicillin compared to control medium minus carbenicillin (Table 6). Kanamycin, however, completely inhibited shoot induction at all levels tested (data not shown). When using 25 mg/L kanamycin, some germinated intact

explants were able to develop a pale green apical meristem. On one occasion, very pale green shoots were observed from the cut end of an explant. The apical meristem and/or any direct shoots produced, however, did not persist. Glufosinate (PPT) completely inhibited imbibed explants from developing direct shoots. No pale green or non-direct shoots were observed at any time and complete decay usually occurred within 10 days. In the experiment to test if PPT inhibited root induction on non-transformed explants containing numerous direct shoots, none of the 24 explants tested produced roots on 20 mg/L PPT. Plants started to brown in five days, and most were completely dead within two weeks.

Seed Sterilization

The tea strainer worked well initially for containing and handling seeds while sterilizing. Corrosion of the strainer while in the acid and incubating in the last water rinse, however, had a negative effect on seed imbibition. There appeared to be a correlation between the age of the strainer, the amount of corrosion present, and the premature rupture of the seeds. The older the strainer, the more corrosion seemed to occur, resulting in premature seed rupture (personal observation) This condition made it difficult to obtain seeds at the correct stage over a defined period of time. It also was not uncommon to have the strainer components disengage due to acid damage. Eliminating the strainer and reducing the acid concentration enabled more consistent and reproducible seed preparation. Decanting the acid and water solutions without losing seeds was difficult. By using 1 ml/L PPM, consistently uniformly imbibed seeds were obtained. This was by far

the easiest and most effective protocol. The seeds were sterilized simply by placing them in 1ml/L PPM and incubating for 15 hours. The seeds could then be removed directly from this initial container and immediately used for obtaining the explant.

Cutting the Explant

Obtaining two split cotyledons, each with an intact hypocotyl, after a single slice with a #10 blade required practice. The slice must be fast, smooth and in the exact spot required. If the seed was not fully imbibed and wet, a smooth cut was difficult to obtain. If the seed coat had already ruptured, it was very difficult or impossible to cut in the required location. Since individual explants were less than 1 mm in size, a binocular microscope was necessary to excise the tissue. Initially an incandescent light source was used, but it was later found that a brighter fiber optic light source reduced eye fatigue. Although labeling each petri dish with a marker was effective, the transparency template greatly enhanced speed and accuracy of cotyledon plating. All petri dishes contained a uniform plastic notch on the bottom dish. Lining up the template with the notch on the dish produced 24 uniform spots for individual cotyledons to be plated. It also made for easy record collection of non-transformed and transformed partners at later dates.

***Agrobacterium* Growth and Storage**

Strain EHA101 was centrifuged and re-suspended with acetosyringone before co-cultivating with germinated cotyledons. Due to the lack of positive results and the fact that many protocols do not re-suspend bacteria, the resuspension step was eliminated. It was

easier to inoculate a single colony of either EHA101 or AGL1 into a flask containing 20 ml LB. The culture was usually ready in 17 hours. Multiple single colonies were sometimes added to increase the rate of growth. Both EHA101:pMON9793 and AGL1:pCP001 appeared and grew similarly on LB agar and LB liquid. Agar streaks grew quickly and produced thick shiny growth. Liquid cultures of both strains contained the same foul odor present at log phase stage. Strain AGL1 that was in -80°C storage for a year grew well after plating on LB agar with the appropriate antibiotics.

Co-Cultivation

Dispensing a micro-drop of *Agrobacterium* was a fast, easy way to introduce the bacteria to the wounded explant. With germinated explants, the drop was applied at the cut region and left for three days. This produced sufficient bacteria growth that explants were rinsed off before plating on selection medium. With the imbibed explants, the micro-drop enclosed the whole explant. The small size of the imbibed cotyledon explants compared to the large volume of stationary inoculmn caused difficulty in removing excess bacteria from explants at the end of co-cultivation. Co-cultivation in cell culture wells alleviated this problem. This approach was more like that of Larkin et al. (1996) in which all cotyledon explants were placed in a petri dish with *Agrobacterium* and periodically agitated. With the wells, each transformed partner genotype received its own well of bacteria. Cotyledons were relatively easy to transport into and out of the wells with practice. After blotting off excess bacteria, the co-cultivating cotyledon explants plated on SH medium did not develop as thick a bacterial growth compared to the micro-drop

method. Cotyledons appeared to have a glaze of thin bacterial growth around them that was rinsed off.

Recovering Plants

Many white clover transformation tests were performed over a 2 ½ year period (Table 7). Non-transformed cultures usually developed green shoots within three weeks. Once placed on root inducing medium, roots formed approximately two weeks later. The transformed cultures were slower in response. Within 7 - 10 days on selection medium, dead explants could be removed. Initially 10 mg/L PPT was used for selection, but no PPT was used in the root inducing medium. Although 10 mg/L PPT completely killed explants not *Agrobacterium* co-cultivated (data not shown), this system produced shoots using co-cultivated explants that appeared to be escapes. The SH+ medium was increased to 20 mg/L PPT and the RI medium was supplemented with 20 mg/L PPT (RI+). In some cases cultures that produced many shoots on SH+, when placed on RI+, developed roots from some shoots but other shoots were killed (Figure 9). Plants that developed roots in either RI or RI+ medium were transplanted into peat pellets and incubated in a mist box. Most plants produced lateral roots that protruded from the sides of the pellet in 5 to 10 days (Figure 10). Although plants rooted quickly in pellets, they were left in the mist box until many roots and new shoot growth had appeared and the plants appeared to be healthy and vigorous. This enabled a gradual adjustment to the greenhouse environment. The plants that were repotted and placed in the greenhouse showed little signs of stress when placed in a normal bench top spot. At the time of transfer to the greenhouse, plants varied in terms

of vigor or amount of growth present. Therefore plants were shaded for two days, in an attempt to temporarily retard transpiration before they were moved to the top of the bench. One genotype using EHA101:pMON9793, the micro-drop and imbibed cotyledons, and ten genotypes using AGL1:pCP001, wells, and imbibed cotyledons were putatively transformed (Table 7). The pMON9793 recovered plant produced aberrant shaped flowers and would not set seed after many hand-crosses. Seed were not produced when this plant was used as either the male or female parent.

Evaluation of Transformed Plants

GUS Histochemical Assays

The *gus* gene was helpful in determining if explants that contained green shoots on selection medium were possible escapes. Both tobacco basic chitinase and mannopine synthase promoters fused to the *gus* gene were effective in detecting enzyme activity when using a thin petiole slice. The single putatively transformed plant (L10) with EHA101:pMON9793 stained positive for the petiole assay in the lab but failed the root assay. Expression was also variable in this plant under greenhouse conditions with complete loss of activity in some tests. Chitinase:*gus* in AGL1:pCP001 produced expected results when using the petiole test, although inconsistent results from the same plant in later tests lead to doubt of stable gene expression and/or stain quality. It was later discovered that clusters of shoots in transformed explants contained non-transformed shoots that developed normally during the whole plant recovery procedure. The GUS root tip assay

with the chitinase promoter was the most reliable indicator of a putatively transformed plant. All white, actively-growing roots on 20 mg/L PPT stained positive in the root tip assay. Staining for three hours at 37°C usually was enough to detect expression of GUS. If the roots were incubated overnight at this temperature, expression was extreme in some situations (Figure 11A). Ten random stolon cuttings were rooted from entry α5#16, the first plant regenerated from the transformation protocol using pCP001, and also thought to contain both non-transformed and transformed sections. Roots from three cuttings stained positive and resisted 200 mg/L PPT, while roots from the other seven cuttings did not stain and were killed by 200 mg/L PPT. Similar results were obtained with many transgenic plants regenerated via tissue culture. A petiole staining test that included progeny from an α5#16 cross, delta-2-#4, delta-1-#11, L10, and controls indicated variable GUS expression from transformants in the greenhouse. Genotype L10 containing the MAS:*gus* construct produced lighter color staining compared to most plants transformed with chitinase:*gus*. Genotype delta-2-#4 contained the weakest GUS expression (Figure 11B) which was also later found to be correlated with reduced resistance to PPT.

Herbicide Painting and Spraying

The PPT painting assay was a good indicator of *bar* gene activity. When the pure ammonium salt was used, it was difficult to keep the solution in sufficient contact with the leaf. As long as a few drops adhered to the tissue, non-transformed leaves developed yellow splotches in those locations and most completely died in a few days. Labeling individual leaves and multiple leaves on the same plant was tedious and usually led to

results that did not indicate a definite status of the plant (transformed, escape, mixed). Misting 20 - 2000 mg/L PPT was an efficient method to determine if a plant was resistant. It was also useful to eliminate non-transformed sections from a transformed plant. When the α5#16 transformed plant contained a full green canopy with many leaves, 200 mg/l PPT was applied. Within one week it was evident that portions of this plant were completely resistant to PPT at this level, while other portions were completely destroyed (Figure 12). After one week, much of the dead plant material was removed, all of which could be traced back to individual crown stolon segments. This scenario of recovering mixed plants was evident in most transformed plants regenerated via tissue culture. The replicated herbicide test performed on entries delta-2#4, α5#16, and delta-2#18, indicated that delta-2#18 was an escape, α5#16 was the most resistant, and delta-2#4 was not as resistant as α5#16. All the control non-transformed partners and delta-2#18 were tolerant to 20 mg/L PPT, but 200 and 2000 mg/L proved to be lethal. None of the treatments were lethal for α5#16 or delta-2#4, however, α5#16 was completely resistant to 200 mg/L and delta-2#4 was not (Table 8). It was later discovered that the delta-2#4 source plant contained portions that were either not transformed or expressing a very low level of resistance. In a later herbicide spray test, delta 1#11, another putatively transformed genotype, appeared almost undamaged when 2000 mg/L PPT was applied indicating this genotype may be expressing the *bar* gene at a higher level than α5#16.

Crossing and Segregation of the Transgene

Eighteen hand crosses were made using α5#16 as a transformed parent. Three other crosses involving delta8#11 and one cross using delta8#16 were also performed. Seed yield per individual flower head ranged from 9 for (Red-Leaf X α5#16) to 87 for (α5#16 X Four-Leaf). Crosses involving Four-Leaf tended to produce the most seed (Table 9). Seven days after application of 50 mg/L PPT to 30 progeny from α5#16 X Four Leaf, a ratio of 17 resistant : 13 lethal ratio was obtained. The null hypothesis of expected Mendelian segregation ratio for disomic inheritance of a single dominant gene (1:1) was tested using the χ^2. A χ^2 value of 0.53; p=0.48 was evidence for a good fit of the data resulting in failure to reject the null hypothesis. The 17 resistant progeny all stained positive for the GUS root tip assay, indicating that both linked genes from the T-DNA insert (*bar*, *gus*) were segregating together. The intensity of staining in the progeny appeared the same as the parent in most cases.

DNA Analysis

The polymerase chain reaction (PCR) was successful at amplifying the internal 418 bp sequence of the *bar* gene from genomic DNA extracted from sample α5#16 (Figure 13) No non-specific DNA amplification was present. Southern blot analysis revealed single positive hybridization bands using DNA extracted from liquid nitrogen frozen plant samples and cut with *Eco*RI. Undigested DNA and DNA cut with *Pst*I yielded hybridization bands of high molecular weight. When DNA was cut with both *Eco*RI and *Pst*I in an attempt to

splice out the intact *bar* gene, two bands were present (Figure 14). Since neither band is the expected size of the *bar* gene (600bp), and the slower band appears to be the same size as DNA cut with *Eco*RI alone, these two bands may be the result of incomplete DNA digestion and are likely the *Eco*RI and *Pst*I fragments expected to contain the *bar* gene. The control containing non-transformed DNA cut with *Eco*RI did not hybridize with the *bar* probe. The lanes that included DNA that was isolated from dehydrated leaves did not produce definite hybridization bands.

Table 5. Comparison for responses to direct shoot induction among white clover cultivars adapted to the southeast United States

Cultivar	N	Percent Response*	Mean # of shoots**
California Ladino	625	51%	2.3A
Osceola Ladino	520	47%	2.2A
Regal Ladino	635	48%	2.1A
Louisiana S1	433	41%	1.8B
Florida Red Leaf	646	39%	1.6B

* Number of genotypes that produced >0 shoots

** Means include zero values. Means with the same letter are not significantly different (α =.05) according to Duncan's LSD test

Table 6. Effect of carbenicillin on direct shoot induction

Treatment	N	Mean
300 mg / L Carbenicillin	155	3.71
0 mg / L Carbenicillin	176	3.52

Table 7. White clover transformation experiments : 1996 - 1998

Test	Date	Explant	Strain:Plasmid	Inoculation	N	# Transformed
	3/6/96 - 6/6/96	germinated-split	EHA101:pMON9793	Micro-Drop		
Test J	2/16/97	Imbibed	EHA101:pMON9793	Micro-Drop	44	
Test K	2/19/97	Imbibed	EHA101:pMON9793	Micro-Drop	21	
Test L	2/25/97	Imbibed	EHA101:pMON9793	Micro-Drop	20	1-#10
Test M	3/26/97	Imbibed	EHA101:pMON9793	Micro-Drop	20	
Test N	3/31/97	Imbibed	EHA101:pMON9793	Micro-Drop	20	
Test O	4/8/97	Imbibed	EHA101:pMON9793	Micro-Drop	20	
Test 1	5/5/97	Imbibed	EHA101:pMON9793	Micro-Drop	25	
Test Z	5/29/97	Imbibed	EHA101:pMON9793	Micro-Drop	15	
Test A	7/7/97	Imbibed	EHA101:pMON9793	Wells	24	
Test H	7/21/97	Imbibed	EHA101:pMON9793	Wells		
Test P1	8/17/97	Imbibed	EHA101:pPSV	Wells	37	
Test P2	8/19/97	Imbibed	EHA101:pPSV	Wells	47	
Test P3	8/22/97	Imbibed	EHA101:pPSV	Wells	47	
Test P4	8/25/97	Imbibed	EHA101:pPSV	Micro-Drop		
Test P5	9/9/97	Imbibed	EHA101:pPSV	Wells	24	
Test P6	9/11/97	Imbibed	EHA101:pPSV	Wells	24	
Test P7	9/16/97	Imbibed	EHA101:pPSV	Wells	24	
Test L1	9/23/97	Imbibed	AGL1:pCP001	Wells	24	
Test A3	9/30/97	Imbibed	AGL1:pCP001	Wells	24	
Test A4	9/30/97	Imbibed	AGL1:pCP001	Wells	24	
Test A6	10/8/97	Imbibed	AGL1:pCP001	Wells	24	
Test A8	10/15/97	Imbibed	AGL1:pCP001	Wells	78	
Test A9	10/30/97	Imbibed	AGL1:pCP001	Wells	24	
Test A10	11/5/97	Imbibed	AGL1:pCP001	Wells	24	
Test B1	11/12/97	Imbibed	AGL1:pCP001	Wells	24	
Test C1	11/12/97	Imbibed	AGL1:pCP001	Wells	24	
Test a1	12/13/97	Imbibed	AGL1:pCP001	Wells	48	
Test a2	12/14/97	Imbibed	AGL1:pCP001	Wells	46	
Test a3	1/20/98	Imbibed	AGL1:pCP001	Wells	22	
Test a4	1/22/98	Imbibed	AGL1:pCP001	Wells	24	
Test a5	1/28/98	Imbibed	AGL1:pCP001	Wells	24	1-#16
Test delta 1	3/10/98	Imbibed	AGL1:pCP001	Wells	24	
Test delta 2	3/10/98	Imbibed	AGL1:pCP001	Wells	24	
Test delta 3	3/11/98	Imbibed	AGL1:pCP001	Wells	24	
Test delta 4	3/11/98	Imbibed	AGL1:pCP001	Wells	24	
Test delta 5	3/18/98	Imbibed	AGL1:pCP001	Wells	24	
Test delta 6	3/24/98	Imbibed	AGL1:pCP001	Wells	24	
Test delta 7	3/24/98	Imbibed	AGL1:pCP001	Wells	24	2-#4,24
Test delta 8	3/25/98	Imbibed	AGL1:pCP001	Wells	24	5-#11,13,16,21,22
Test delta 9	3/25/98	Imbibed	AGL1:pCP001	Wells	24	
Test delta 10	3/31/98	Imbibed	AGL1:pCP001	Wells	24	
Test W2	4/7/98	Imbibed	AGL1:pCP001	Wells	24	
Test W3	4/7/98	Imbibed	AGL1:pCP001	Wells	24	
Test W4	4/8/98	Imbibed	AGL1:pCP001	Wells	24	
Test W5	4/8/98	Imbibed	AGL1:pCP001	Wells	24	

Table 8. Response of transformed white clover to the herbicide PPT.

Entry	ID	Replication	mg/L PPT	Rating (0-5)*
1	delta2#4-NT	1	0	0
2	delta2#4-NT	2	0	0
3	delta2#4-NT	1	20	2
4	delta2#4-NT	2	20	2
5	delta2#4-NT	1	200	5
6	delta2#4-NT	2	200	5
7	delta2#4-NT	1	2000	5
8	delta2#4-NT	2	2000	5
9	delta2#4-T	1	0	0
10	delta2#4-T	2	0	0
11	delta2#4-T	1	20	1
12	delta2#4-T	2	20	0
13	delta2#4-T	1	200	4
14	delta2#4-T	2	200	1
15	delta2#4-T	1	2000	2
16	delta2#4-T	2	2000	1
17	a5#16-NT	1	0	0
18	a5#16-NT	2	0	0
19	a5#16-NT	1	20	4
20	a5#16-NT	2	20	3
21	a5#16-NT	1	200	5
22	a5#16-NT	2	200	5
23	a5#16-NT	1	2000	5
24	a5#16-NT	2	2000	5
25	a5#16-T	1	0	0
26	a5#16-T	2	0	0
27	a5#16-T	1	20	0
28	a5#16-T	2	20	0
29	a5#16-T	1	200	0
30	a5#16-T	2	200	0
31	a5#16-T	1	2000	2
32	a5#16-T	2	2000	1
33	delta2#18-NT	1	0	0
34	delta2#18-NT	2	0	0
35	delta2#18-NT	1	20	4
36	delta2#18-NT	2	20	4
37	delta2#18-NT	1	200	5
38	delta2#18-NT	2	200	5
39	delta2#18-NT	1	2000	5
40	delta2#18-NT	2	2000	5
41	delta2#18-T	1	0	0
42	delta2#18-T	2	0	0
43	delta2#18-T	1	20	3
44	delta2#18-T	2	20	3

Table 8–continued.

Entry	ID	Replication	mg/L PPT	Rating (0-5)*
45	delta2#18-T 1		200	5
46	delta2#18-T 2		200	5
47	delta2#18-T 1		2000	5
48	delta2#18-T 2		2000	5

* 0 = no visable leaf damage, 1-4 = increasing levels of damage, 5 = Complete plant death

Table 9. Seed yield from various white clover hand crosses

Cross	Number of Seed/Inflorescence
Four Leaf X α5#16	82
Four Leaf X α5#16	40
Red Leaf X α5#16	19
Red Leaf X α5#16	9
Red Leaf X α5#16	36
Red Leaf X α5#16	44
Red Leaf X α5#16	38
Red Leaf X α5#16	33
α5#16 X Red Leaf	35
α5#16 X Four Leaf	46
α5#16 X Red Leaf	34
α5#16 X Red Leaf	50
α5#16 X?	50
a5#16 X Four Leaf	87
α5#16 X Red Leaf	52
α5#16 X Red Leaf	61
α5#16 X Red Leaf	56
α5#16 X Red Leaf	32
Delta8#11 X Four Leaf	20
Delta8#11 X Four Leaf	23
Four Leaf X Delta8#11	18
Four Leaf X Delta8#16	17

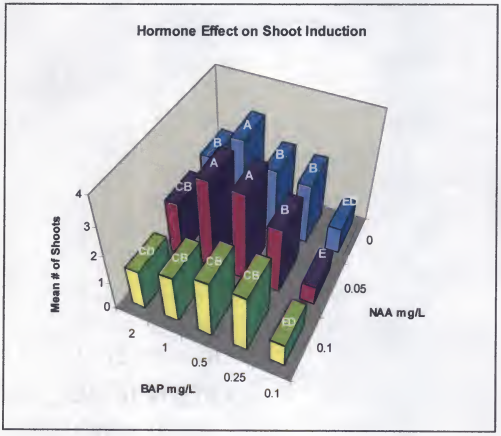

Figure 5. Effect of the hormones BAP and NAA on direct shoot induction. Mean # of shoots includes genotypes that produced zero shoots. Treatments with different letters are significantly different (α=.05) according to DNMRT.

Figure 6. Direct shoots forming on 21 day old explants obtained from germinated white clover seedlings.

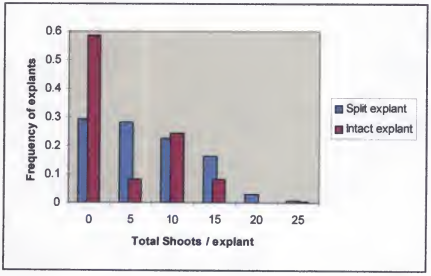

Figure 7. Histogram comparing split and intact explants for response to shoot induction.

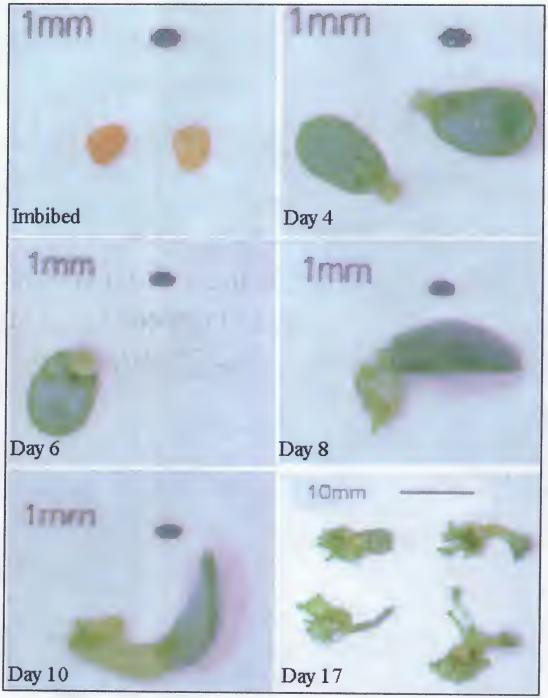

Figure 8. Stages of shoot development using the imbibed seed as an explant source

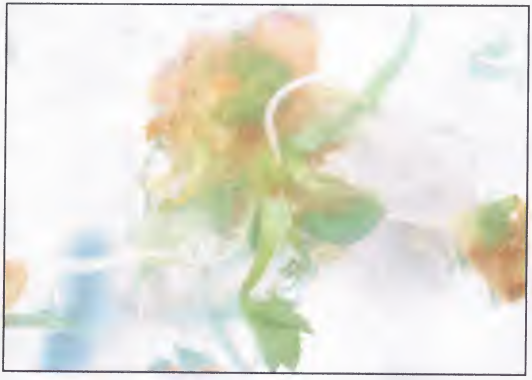

Figure 9. Root emerging from PPT resistant shoot while other non-transformed shoots are killed.

Figure 10. Rooted plant in peat pellet and many plants incubating in a mist-box.

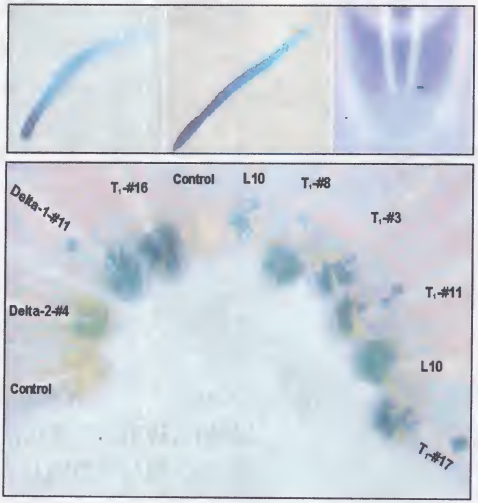

Figure 11. GUS histochemical staining of transformed white clover tissue. Root assay (Top) with plants transformed with AGL1:pCP001. Petiole assay (Bottom) on genotype L10 transformed with EHA101:pMON9793 and an assortment of genotypes transformed with AGL1LpCP001.

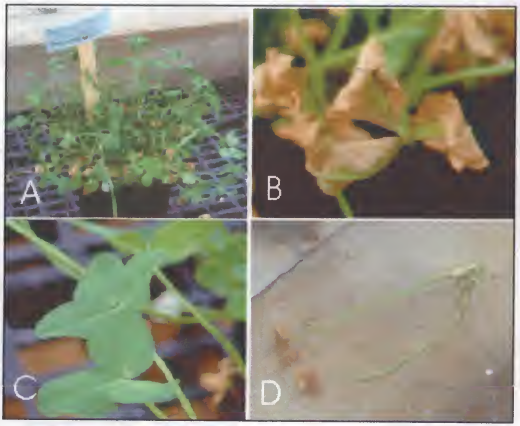

Figure 12. Effect of 200 mg/L PPT on partially transformed plant α5#16. A) Full plant with partially resistant and susceptible segments. B), C) Close view of susceptible and resistant leaves respectively. D) Susceptible segments tracing back to a single stolon.

Figure 13. PCR amplification of the *bar* gene from transformed genomic DNA. Lanes 1,2,3 transformed a5#16 genomic DNA diluted 1/100,1/500, 1/1000 respectively. Lane 4 and 5 a5#16 non-transformed genomic DNA diluted 1/500, 1/1000. Lanes 6 and 7 BAR gene diluted 1/100 and 1/10,000. The identical monochrome image on the right was included for enhanced visibility of amplification bands.

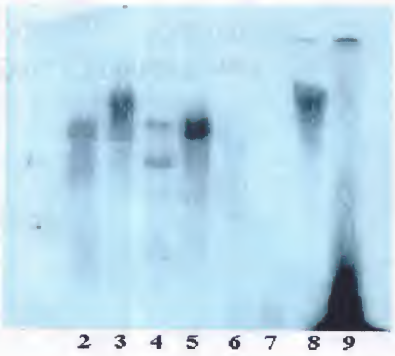

Figure 14. Southern blot of white clover genomic DNA. α5#16 DNA cut with *Eco*RI (lanes 2 and 5), *Pst*I (lane 3), *Eco*RI and *Pst*I (lane 4), and undigested (lane 8). DNA extracted from dehydrated leaves and cut with *Eco*RI (lane 6) and non-transformed DNA (lane 7). The 600 bp bar gene (lane 9) ran off the gel.

DISCUSSION

White Clover Regeneration

Results indicated that medium containing 0.05 mg/L NAA and 1 mg/L BAP was most effective at inducing direct shoots with germinated seedling explants. These experimental results indicated that the cotyledon stalk, specifically the region that unites with the hypocotyl, is the primary tissue responsible for shoot induction. Splitting the cotyledons and plating them abaxial side down allowed direct contact between this region and the medium. Intact explants cut slightly proximal of the hypocotyl region had a much lower chance of responding due to lack of contact between medium and receptive tissue. The fact that Voisey et al (1994) did not use this more responsive explant for *Agrobacterium* transformation, instead using the intact explant to recover a transformed apical meristem at low frequency, leads to uncertainty of the compatibility of these regeneration systems with *Agrobacterium* transformation. Although it was more difficult to obtain and manipulate explants from imbibed seeds, this protocol was more effective and faster in time required for direct shoot induction compared to germinated explants. Another benefit was the shorter seed preparation time, 15 hours compared to five days on germination medium. Both protocols utilize the hypocotyl region that connects with the

cotyledon stalk, which seems to be more responsive to shoot induction at an embryonic stage. It is not clear whether it is the specific stage of the explant tissue, the medium, the growth regulators, or interactions that are responsible for the high frequency genotype independent shoot induction with imbibed seeds.

Unlike inbred lines, open-pollinated cultivars are a composite of genotypes with all desirable genes (traits) theoretically maintained at constant frequencies over generations. Single copy integration of a transgene into a large number of agronomic genotypes followed by selection of the best transformed genotype in terms of transgene expression will be prerequisites for cultivar development in open-pollinated species. If many independent transformed genotypes with different genome integration sites or greater than single copy per genome plants are used as parents, problems in trait stabilization and genetic load may occur in future generations. Genotypes derived from material selected for regeneration usually lack agronomic potential. Although transformation may be efficient, these genotypes would not make suitable founding parents. The real utility with methods that are genotype independent will be the ability to transform many genotypes followed by selection of the best individual in terms of overall agronomic potential, insert copy number, and transgene expression. This desirable transformed plant can then be used as a parent in cultivar development or improvement.

White Clover Transformation

The majority of transformed plants were recovered from tests using *Agrobacterium* strain AGL1 harboring plasmid pCP001. AGL1 and EHA101 are very similar strains of *Agrobacterium*, both derived from the wild-type C58. Plasmid pCP001 and pMON9793 differ in certain key aspects. The 35S promoter controlling transcription of the *bar* selectable marker gene is thought to be stronger than the NOS promoter controlling *nptII* on pMON9793. Although both strains produced escapes, kanamycin selection produced more shoots that slowly died in contrast to PPT. Most escapes with strain AGL1 occurred before PPT was added to the root inducing medium and 10 mg/L PPT was used in the shoot inducing medium. Since PPT is not thought to be systemic within the plant, a few non-transformed cells surrounded by transformed cells may allow shoot initials to form under selection. If these shoots are not in good contact with the medium, a cluster of shoots from a single explant may contain some non-transformed initial shoots. This situation was evident in all transgenic plants regenerated via tissue culture. It is not clear if the mixed plants were recovered due to transformed shoots detoxfying PPT around non-transformed shoots, lack of efficient contact of shoots and PPT containing medium, concentration of PPT or other unknown factors. Problems could arise if these initial laboratory derived plants not only contain portions that are not transformed but if transformed sections contain multiple different T-DNA insertions. Root specific GUS staining was linked to herbicide resistance in mixed plants. Entry α5#16 was recovered

without PPT in the rooting medium and contained mixed non-transformed and transformed segments originating from distinct crown sections. Since mixed plants still occured with PPT in the rooting medium, unless individual shoots are removed and maintained at a very early age, mixed plants may be inevitable. Spraying PPT on mixed plants will eliminate non-transformed segments but will not solve the problem of multiple, different integration sites. After being screened by spraying 200mg/L PPT, entry α5#16 indicated single copy integration based on Southern blot data and sexual transmission segregation data. Although single copy integration was present in α5#16, it is unknown if this situation will occur in all other transgenic plants. If this escape scenario consistently occurs at 100% frequency, it may be possible to recover the non-transformed control plants by detecting them with a leaf painting or mild PPT spraying assay from source plants. This would eliminate the extra handling and maintenance required in the lab to recover the non-transformed control plant.

Once the timing for seed sterilization and swelling was more predictable, it was easier to synchronize log phase bacteria and explant preparation. The cell culture well plates were beneficial in maintaining the identity of individual cotyledons while also providing liquid swirling type exposure to *Agrobacterium*. Recovering the non-transformed control plant required more handling of the partner explant being co-cultivated. The extra handling at a young stage, however, may lower the chances for direct shoot induction. Assuming setup timing of all required components for cutting and co-cultivation is achieved and the cotyledons are obtained intact with an undamaged hypocotyl, this transformation method appears to be suitable for transgenic cultivar development in white clover.

Breeding Transgenic White Clover

Most of the literature on plant genetic engineering pertains to new protocols for gene integration or novel genes that may have great agronomic potential. Rarely is the transfer of the novel gene, now located in a single genome, into an existing cultivar discussed. If the cultivar is released as an inbred line or is asexually propagated then, in theory, a single elite transformed plant from that cultivar can be amplified via self pollination and/or cloning and released. For outcrossing, highly heterozygous species like white clover, a different breeding strategy may be required. Voisey et al., 1994 described a method of breeding transgenic white clover. Their first step was to produce plants homozygous for the new insert by crossing primary transformants with elite genotypes and then intermating transgenic plants from the F_1 population. Transgenic white clover cultivars can be produced by selecting intermating F_2 plants homozygous for the transgene and with good general combining ability to intermate for 5-6 generations. They state that the transgenic cultivar will, therefore, be comprised of individuals which are homozygous for the introduced gene and should deliver the agronomic performance which characterized the genotypes from which they were derived. Two problems may occur with this proposed breeding method. First, by crossing more than a single primary transformed plant, the transgene will most likely be housed at more than one locus. In later generations of random mating, this could cause problems with genetic load and trait stability. To ensure stable permanent expression of the new gene, it should be confirmed that the founding parent has a single hemizygous locus with the insert. It should then be confirmed that in

the F_2 generation the insert segregates in a Mendelian fashion and that homozygous and hemizygous individuals do not differ in expression of the new transgene. Then, in theory, all individuals in later generations should only contain the insert at a single locus in either a hemizygous or homzygous functioning state. If multiple independent transformed plants are used, the number of unlinked repetitive loci containing the transgene will increase with random mating. Due to new homology in different regions of the genome, genetic recombination during meiosis may occur between non-homologous chromosomes causing cytogenetic alterations such as duplications and deletions. With a high copy number of the transgene, the chances of a gene silencing effect at the DNA, RNA, or protein level may increase. Secondly, the Voisey et al., 1994 breeding approach does not appear to maximize heterozygosity which is essential for optimum white clover population performance. A different breeding strategy that may address these drawbacks is discussed in the next paragraph.

The main objectives of this proposed breeding method are to maintain all beneficial qualities of the original cultivar, maximize heterozygosity, and ensure that the new transgene is maintained at a sufficient frequency in future generations. This breeding strategy is basically a backcross approach for an open pollinated species. A single transformed plant would be used to move the new gene into the existing cultivar. It is critical that this founding parent be free of defects due to DNA insertion effects or somaclonal variation. The availability of a non-transformed control plant that has the same genetic background and was regenerated *in vitro* substantially aids in the selection process. Although it is more tedious in the lab to transform and maintain material when recovering

control plants, the assurance that this crucial selection decision is made correctly makes it worthwhile. It should be beneficial to compare a population of transformed/non-transformed partners and then select the single best transformed plant rather than to select the best plant from a random group of 200 transformants, considering that after the first cross, all the progeny are half-sibs related to this initial parent. This breeding strategy, described here with cultivar Osceola, but applicable to any white clover cultivar, requires a minimum of 50 paired genotypes containing a gene for easy large scale selection of transgenic plants in later generations, such as herbicide resistance. Replicated experiments of transformed and non-transformed plants to compare for differences in yield, vigor, flowering characteristics, and persistence should be completed immediately after plants are available. The single best transformed plant should be selected and multiplied asexually. In an ideal white clover seed production area that also provides an environment suitable for selection, randomly plant 30 transformed clones in the field in a 1 transformed clone : 10 random Osceola plants proportion, with all seed harvested (T_1). This is done in to avoid the development of a population containing a single cytoplasm. If seeds were solely harvested from transformed clones, then all future generations would contain an identical cytoplasm. The remainder of the breeding strategy is designed to reduce the inbreeding coefficient of the population being selected, recover the Osceola phenotype, and increase the gene frequency of the new gene(s). The T_1 generation should be grown as spaced plants and sprayed with herbicide to eliminate any non-transformed individuals before selection. Re-select the best transformed plants, at least 50 from no less than 1000 genotypes, if space and labor are available. Place these transformed plant selections back

into Osceola at random locations and harvest seed (T_2). Repeat the selection and reintroduction of transformed plants back into Osceola cultivar until the group of transformed plants under selection exhibits all desirable traits of Osceola in addition to the transgene. Further selections, if required, should be in the form of mass selection and originate from seed produced by intercrossing the last transformed generation evaluated.

Patent Problems

The future is uncertain in terms of who will be able to develop and release transgenic cultivars. There are now many confirmed and efficient transformation protocols for most valuable species, however, apparently each component required in the complete transformation system has been patented. For example, the biolistic bombardment and *Agrobacterium* transformation protocols are patented. The patent source depends on the crop. For example, if you want to transform turf grass with a biolistic approach, then Scotts (The Scotts Company, Ohio, USA) holds the rights. Patents for many of the best plant promoters, including the 35S, are held by Monsanto. The trend appears to be that a few large companies are purchasing smaller bio-tech and seed companies in order to produce transgenic cultivars without any patent concerns. University scientists are using many of these components in order to develop new transformation protocols and investigate transgenic gene expression. Currently it would be very difficult to develop a system that does not rely on any patented components. Universities may soon discover that it is too costly to develop transgenic cultivars because of licensing costs to holders of

patents. The convoluted legal quagmire of this situation may have an impact in the use of technology and the development of cultivars in the university system.

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BIOGRAPHICAL SKETCH

Jason James Goldman was born to Robert and Johanna Goldman in Pequannock, New Jersey. He graduated from Wayne Valley High School in 1988. Jason attended Rutgers University, Cook College in New Brunswick, New Jersey majoring in plant science. He graduated in 1992 from Rutgers University receiving a Bachelor of Science degree. In 1994, Jason completed his master's degree in plant breeding at Texas A&M University, College Station, Texas. In 1998, he will complete work on his doctorate degree in plant breeding.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

David S. Wofford, Chair
Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Kenneth H. Quesenberry
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Rex L. Smith
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1998

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